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A Serum Response Factor-Dependent Transcriptional Regulatory Program Identifies Distinct Smooth Muscle Cell Sublineages

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The SM22α promoter has been used as a model system to define the molecular mechanisms that regulate smooth muscle cell (SMC) specific gene expression during mammalian development. The SM22α gene is expressed exclusively in vascular and visceral SMCs during postnatal development and is transiently expressed in the heart and somites during embryogenesis. Analysis of the SM22α promoter in transgenic mice revealed that 280 bp of 5′ flanking sequence is sufficient to restrict expression of the *lacZ* reporter gene to arterial SMCs and the myotomal component of the somites. DNase I footprint and electrophoretic mobility shift analyses revealed that the SM22α promoter contains six nuclear protein binding sites (designated smooth muscle elements [SMEs] -1 to -6, respectively), two of which bind serum response factor (SRF) (SME-1 and SME-4). Mutational analyses demonstrated that a two-nucleotide substitution that selectively eliminates SRF binding to SME-4 decreases SM22α promoter activity in arterial SMCs by approximately 90%. Moreover, mutations that abolish binding of SRF to SME-1 and SME-4 or mutations that eliminate each SME-3 binding activity totally abolished SM22α promoter activity in the arterial SMCs and somites of transgenic mice. Finally, we have shown that a multimerized copy of SME-4 (bp −190 to −110) when linked to the minimal SM22α promoter (bp −90 to +41) is necessary and sufficient to direct high-level transcription in an SMC lineage-restricted fashion. Taken together, these data demonstrate that distinct transcriptional regulatory programs control SM22α gene expression in arterial versus visceral SMCs. Moreover, these data are consistent with a model in which combinatorial interactions between SRF and other transcription factors that bind to SME-4 (and that bind directly to SRF) activate transcription of the SM22α gene in arterial SMCs.

Smooth muscle (SM) cells (SMCs) play important roles in organ systems throughout the body, subserving such diverse functions as modulation of arterial tone, controlling gastrointestinal motility, and regulating airway resistance. The unique functional capacities of this muscle cell lineage are determined by the expression of distinct sets of tissue-specific genes encoding contractile proteins, cell surface receptors, and intracellular enzymes (48). A feature that distinguishes the SMC lineage(s) from the striated muscle cell lineages is the capacity of SMCs to reversibly modulate their phenotype during postnatal development (13, 39, 49, 62). For example, vascular SMCs located in the arterial tunica media are maintained in the resting, or G_0/G_1, phase of the cell cycle and express high levels of contractile protein isoforms (55). However, in response to vessel wall injury and the concomitant release of growth factors, these cells reenter the cell cycle, proliferate, and modulate their phenotype from primarily contractile to primarily synthetic (13, 49, 62). This phenotypic modulation has been implicated in the pathogenesis of cardiovascular diseases, including atherosclerosis and restenosis after balloon angioplasty (55, 60).

In contrast to the striated muscle cell lineages, relatively little is currently understood about the molecular mechanisms that control SMC lineage specification, differentiation, and phenotypic modulation. This is due, in part, to the poorly understood lineage relationships of SMCs, which appear to develop from multiple locations throughout the embryo, as well as, the relative paucity of SMC-specific markers (for a review, see reference 48). One approach to understanding the molecular mechanisms that regulate SMC development and differentiation is to identify and characterize the *cis*-acting sequences and *trans*-acting factors that regulate SMC-specific gene expression. This approach has provided fundamental insights into the molecular programs that regulate both skeletal and cardiac muscle cell differentiation (for review, see references 46 and 65). However, the following observations suggest that a distinct molecular program must control SMC differentiation. (i) SMCs express a unique developmentally regulated set of contractile protein isoforms, including SM-myosin heavy chain, calponin, and γ-enteric actin, which are not expressed in the striated muscle cell lineages (for review, see reference 48). (ii) SMCs develop in a temporal and spatial pattern distinct from that of skeletal or cardiac myocytes during embryonic development (14, 22, 35, 50). (iii) In contrast to both skeletal and cardiac myocytes, SMCs retain the capacity to both proliferate and express contractile protein isoforms during postnatal development (48, 60, 62). (iv) The basic helix-loop-helix (bHLH) family of myogenic transcription factors are not expressed in SMCs, and mice harboring null mutations in MyoD, Myf-5, and myogenin exhibit normal development of SMC-containing tissues (18, 41, 56).

Current developmental paradigms suggest that lineage determination and cellular differentiation are ultimately controlled by the expression of lineage-specific transcription factors (for review, see references 45 to 47). However, to date, no SMC lineage-specific transcription factors have been identified. Thus, it remains unclear whether common, overlapping, or distinct sets of *cis*-acting sequences and *trans*-acting factors

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regulate SMC and striated muscle (cardiac or skeletal)-specific transcription. Interestingly, several members of the MADS (named for family members MCM1-Agamous-Deficiens-serum response factor [SRF]) box family of transcription factors (for review, see reference 66), including MEF2 and SRF, have been implicated in SMC differentiation. Lilly et al. reported that a null mutation of the *Drosophila* MADS box transcription factor gene, coding for the D-MEF2 protein, resulted in the failure of somatic, cardiac, and visceral muscles to differentiate (29). These data suggest that this evolutionarily conserved transcription factor may play a critical role in coordinating the differentiation of all three muscle cell lineages. It is also noteworthy that both the SM–α-actin and SM-myosin heavy chain promoters contain functionally important binding sites for the MADS box transcription factor, SRF (2, 3, 21). This transcription factor appears to play an important role in cell proliferation and differentiation (20, 67). SRF is required for transcriptional regulation of growth factor-inducible genes, including the proto-oncogene c-*fos* and muscle-specific actins (31, 36, 38, 40). Interestingly, full transcriptional activity from the c-*fos* serum response element (SRE), or CArG box, requires accessory factors that bind to the SRF-SRE complex, including members of the *ets* family of transcription factors, Elk-1, SAP-1a, SAP-1b, SAP-2, and ERP-1, each of which is expressed in a lineage-restricted fashion (8, 32, 67). In addition to functionally synergizing with SRF, these accessory proteins are required for transcriptional activation in response to mitogen-activated protein (MAP) kinases (54) suggesting that the SRF-SRE-accessory factor ternary complex may serve to link events at the cell surface with nuclear transcription.

Because of its SMC-specific pattern of expression, we have used the murine SM22α gene as a model system with which to examine the molecular mechanisms regulating gene expression in SMCs (10, 12, 27, 44, 63). SM22α is a 22-kDa protein with structural homology to the vertebrate thin filament myofibrillar regulatory protein calponin (64) and the *Drosophila* muscle protein mp20 (1). It contains two potential calcium binding domains that are oriented in an HLH, or EF-hand, conformation (23). Previous studies have demonstrated that the SM22α gene product is abundantly expressed in SMCs and is one of the earliest markers of the SMC lineage (10). Of note, the SM22α gene is expressed at high levels in contractile SMCs within the arterial media, but not within synthetic SMCs located within human atheromatous plaques (61). We have reported previously that the murine SM22α gene is composed of five exons spanning 6.2 kb of genomic DNA. In addition, we have demonstrated that SM22α gene expression is controlled by an SMC-specific promoter located within the immediate 5′ flanking region (bp −441 to +41) of the gene (63).

In the studies described in this report, we have used both transient transfections and the production of transgenic mice to identify the *cis*-acting elements that control SM22α gene expression in arterial SMCs. In addition, we have performed nuclear protein binding assays to define the *trans*-acting factors that regulate SM22α gene expression. Finally, we have performed mutational analyses to examine the functional role of nuclear protein binding sites in the SM22α promoter. These studies have demonstrated that the 280-bp SM22α promoter is sufficient to restrict expression of a heterologous reporter gene to arterial SMCs and the developing somites in transgenic mice. The SM22α promoter contains six nuclear protein binding sites, designated SM elements (SMEs) -1 to -6, respectively, that bind SRF, YY1, and Sp1, as well as, several potential novel *trans*-acting factors. Activity of the SM22α promoter in arterial SMCs, both in vitro and in vivo, is critically dependent upon the SME-4 nuclear protein binding site which binds *trans*-acting factors, including SRF and YY1. These data are consistent with a model wherein combinatorial interactions between SRF and other transcription factors that bind to SME-4 (and directly to SRF) regulate SM22α gene expression in arterial SMCs. Moreover, these data strongly suggest that distinct transcriptional regulatory programs distinguish tissue-specific subsets of SMCs.

MATERIALS AND METHODS

Cell culture. Primary rat aortic SMCs were isolated from 12- to 16-week-old Sprague-Dawley rats and grown as described previously (4, 11). Virtually all cells stain positive for expression of SM–α-actin by this method (4). In all experiments, only second- or third-passage SMCs were utilized. The rat aortic SMC line A7r5, mouse NIH 3T3 and C3H10T1/2 fibroblasts, mouse EL4 T cells, human clone 13 B cells, and human HepG2 hepatocellular carcinoma-derived cells were grown as described previously (37).

DNase I footprinting. Nuclear extracts were prepared from the SMC line A7r5 (which expresses high levels of SM22α mRNA [63]) and NIH 3T3 cells as described previously (53). Three genomic subfragments (bp −441 to −256, −256 to −89, and −89 to +41) spanning the 482-bp SM22α promoter were analyzed. DNase I footprint analyses were performed with 100 to 150 μg of nuclear extracts and the end-labeled sense and antisense strands of the murine SM22α promoter as described previously (51). Standard Maxam and Gilbert (G + A) sequencing reactions were run in parallel to identify the protected sequences.

EMSAs. Nuclear extracts were prepared from low-passage-number primary rat aortic SMCs, A7r5 cells, NIH 3T3 cells, C3H10T1/2 cells, and EL4 cells as described by Dignam et al. (9). Electrophoretic mobility shift assays (EMSAs) were performed in 0.25× TBE (1× TBE is 100 mM Tris, 100 mM boric acid, and 2 mM EDTA) as described previously (19). The following complementary oligonucleotides (corresponding to each nuclear protein binding site identified by DNase I footprint analysis or nuclear protein binding sites containing the specific mutations indicated [mutated nucleotides are underlined]) were synthesized with *Bam*HI and *Bgl*II overhanging ends: SME-1, 5′ AAGGAAGGGTTTCAGGGT CCTGCCCATAAAAGGTTTTTCCCGGCCGC 3′; μSME-1, 5′ AAGGAAGG GTTTCAGGGTCCTGCCCATAGATCTTTTTTCCCGGCCGC 3′; SME-3, 5′ CTCCAAAGCATGCAGAGAATGTCTCCGGCTGCCCCCG 3′; μSME-3, 5′ CTCGGATCCATGCTAGCAATGAATTCGGCTGCCCCCG 3′; μSME-3/ YY1, 5′ CTCCAAAGCATGCAGAGAATGAATTCGGCTGCCCCCG 3′; SME-4, 5′ TCCAACTTGGTGTCTTTCCCCAAATATGGAGCCTGTGTGG AGTG 3′; μSME-4, 5′ TCCAACTTGGTGTCTTTCCCCAAGGATCCAGCC TGTGTGGAGTG 3′; μSME-4/SRF, 5′ TCCAACTTGGTGTCTTTCCCCGG ATATGGAGCCTGTGTGGAGTG 3′; μSME-4/YY1, 5′ TCCAACTTGGTG TCTTTCCCCAAATTAGGAGCCTGTGTGGAGTG 3′; SME-6, 5′ GGACG GCAGAGGGGTGACATCACTGCCTAGGCGGCCG 3′; μSME-6/CREB, 5′ GGACGGCAGAGGGGATCCATGCCTGCCTAGGCGGCCG 3′; μSME-6/ YY1, 5′ GGACGGCAGAGGGGTGACGCGTCTGCCTAGGCGGCCG 3′; and Sp1, 5′ CTGGCTAAAGGGGCGGGGCTTGGCCAGCC 3′.

For cold competition experiments, 5 to 75 ng of unlabeled competitor oligonucleotide was included in the binding reaction mixture. For antibody supershift experiments, 1 μl of either rabbit preimmune, affinity-purified rabbit or mouse immunoglobulin G (IgG) (Santa Cruz), α-SRF rabbit polyclonal antiserum (Santa Cruz; sc-335X), α-Sp1 rabbit polyclonal IgG (Santa Cruz; sc-059X), or α-YY1 rabbit polyclonal IgG (Santa Cruz, sc-281X) was incubated with the indicated nuclear extract at 4°C for 20 min prior to the binding reaction as described previously (19).

Plasmids. To assess the function of nuclear protein binding sites identified within the SM22α promoter, a series of SM22α mutant promoter-luciferase reporter plasmids were generated by PCR-mediated site-directed mutagenesis as described previously (37). The pGL2-Basic (Promega) promoterless luciferase reporter plasmid and the pMSVβgal reference plasmid have been described previously (51). The pcDNASRF and pcDNAYY1 expression plasmids contain the cDNAs encoding the human SRF and YY1 proteins, respectively, subcloned into the polylinker of pcDNA3 (Invitrogen). The p-441SM22luc plasmid contains the 482-bp (bp −441 to +41) SM22α genomic subfragment subcloned immediately 5′ of the luciferase reporter gene and has been described previously (63). The p-90SM22luc reporter plasmid contains the 131-bp murine SM22α promoter (bp −90 to +41) subcloned into *Mlu*I/*Hin*dIII-digested pGL2-Basic. The p-90SME-4X4luc reporter plasmid is identical to the p-90SM22luc plasmid, except that four copies of a double-stranded oligonucleotide corresponding to SME-4 and 18 bp of flanking sequence (bp −190 to −110) was subcloned into the *Sac*I/*Kpn*I-digested (immediately upstream of the minimal SM22α promoter) p-90SM22luc reporter plasmid. The following SM22α mutant promoter-luciferase reporter plasmids were generated and named according to the specific nuclear protein binding site within the promoter that was mutated (mutated nucleotides within each nuclear protein binding site are underlined): p-441SM22μSME-1 (5′ AAGGAAGGGTTTCAGGGTCCTGCCCATAGATC TTTTTTCCCGGCCGC 3′), p-441SM22μSME-3 (5′ CTCGGATCCATGCTA GCAATGAATTCGGCTGCCCCCG 3′), p-441SM22μSME-3/YY1 (5′ CTCC AAAGCATGCAGAGAATGAATTCGGCTGCCCCCG 3′), p-441SM22μSME-4

TABLE 1. Functional analysis of the SM22α promoter in transgenic mice

Vector	No. of founders	Copy no.	Tissue specificity of transgene expression
−441SM22-lacZ	6	5–160	β-Galactosidase activity was observed in the dorsal aorta and branch arteries, myotomal component of the somites, bulbo-truncus (cardiac outflow tract), and bulbo-cordis (future right ventricle of the heart).
−280SM22-lacZ	7	2–34	A tissue-restricted pattern of β-galactosidase activity identical to that obtained in −441SM22-lacZ transgenic mice was observed, except a patchy-blue appearance was demonstrated in the dorsal aorta and branch arteries.
−5000SM22-lacZ	2	1–22	β-Galactosidase activity was observed in the dorsal aorta and branch arteries, myotomal component of the somites, bulbo-truncus, and bulbo-cordis.
−441SM22μCARG-lacZ	12	1–730	No β-galactosidase activity was observed in the arteries, myotomal component of the somites, or heart.[a]
−441SM22μSME-3-lacZ	2	2–5	No β-galactosidase activity was observed in the arteries, myotomal components of the somites, or heart.

[a] In one line of −441SM22μCARG-lacZ mice, low-level β-galactosidase activity was detected in the cardiac outflow tract.

(5′ TCCAACTTGGTGTCTTTCCCCAAGGATCCAGCCTGTGTGGAGTG 3′), p-441SM22μSME-4/SRF (5′ TCCAACTTGGTGTCTTTCCCCGGATATG GAGCCTGTGTGGAGTG 3′), p-441SM22μSME-4/YY1 (5′ TCCAACTTGG TGTCTTTCCCCAAATTAGGAGCCTGTGTGGAGTG 3′), and p-441SM22 μSME-6/YY1 (5′ GGACGGCAGAGGGGTGACGCGTCTGCCTAGGCGG CCG 3′). In addition, several SM22α promoter-luciferase reporter plasmids were subcloned that contain mutations in multiple *cis*-acting elements. p-441SM22 μCArG contains the mutations described above in the SME-1 and SME-4 sites (which abolish SRF binding activity), and p-441SM22μYY1 contains the mutations described above that selectively eliminate binding of YY1 to SME-3, -4, and -6. Each PCR-generated SM22α promoter mutant was confirmed by DNA sequence analyses as described previously (53).

To identify functionally important *cis*-acting elements that control the expression of the SM22α gene in vivo, transgenic vectors were cloned that encode the bacterial *lacZ* reporter gene under the transcriptional control of the native or mutated SM22α promoter fragments. The p-5000SM22-lacZ, p-441SM22-lacZ, p-441SM22μCArG-lacZ, p-441SM22μSME-3-lacZ, and p-280SM22-lacZ plasmids contain the 5-kb SM22α promoter, the 441-bp SM22α promoter, the 441-bp SM22α promoter with mutations in SME-1 and SME-4, the 441-bp SM22α promoter with mutations in SME-3, and the 280-bp SM22α promoter, respectively, subcloned immediately 5′ of the bacterial *lacZ* reporter gene in a modified pBluescript IIKS (Stratagene) plasmid.

Transfections and luciferase assays. Cultures of primary rat aortic SMCs were transfected with 100 μg of Lipofectin reagent (Gibco), 15 μg of luciferase reporter plasmid, and 5 μg of the pMSVβgal reference plasmid as described previously (58). For the cotransfection experiments, primary rat aortic SMCs were transfected with between 0 and 20 μg of either the pcDNAYY1 expression plasmid or the parent plasmid pcDNA3 (the total amount of expression plasmid was held constant at 20 μg), 5 μg of the p-441SM22luc reporter plasmid, and 5 μg of the pMSVβgal reference plasmid with the Lipofectin reagent as described above. A7r5 cells and NIH 3T3 cells were transfected with 50 and 20 μg, respectively, of Lipofectin reagent, 15 μg of luciferase reporter plasmid, and 5 μg of the pMSVβgal reference plasmid. HepG2 cells were transfected with 360 μg of Lipofectamine reagent (Gibco), 26 μg of luciferase reporter plasmid, and 9 μg of the pMSVβgal reference plasmid. Clone 13 cells were transfected by electroporation (0.28 kV and 960 μF) with 50 μg of luciferase reporter plasmid and 7 μg of the pMSVβgal reference plasmid. Forty-eight hours after transfection, cell lysates were prepared and normalized for protein content (Bio-Rad) and the expressed luciferase activities (light units) were corrected for variations in transfection efficiencies as determined by assay of cell extracts for β-galactosidase activities. All experiments were repeated at least three times to ensure reproducibility. Data are expressed as relative luciferase activities ± standard errors.

Transgenic mice. Transgenic mice harboring the −5000SM22-lacZ, −441SM22-lacZ, −441SM22μCArG-lacZ, −441SM22μSME-3-lacZ, −441SM22μCArG-lacZ, and −280SM22-lacZ transgenes were produced according to standard techniques as previously described (34). To identify transgenic founder mice, Southern blot analyses were performed with the radiolabeled *lacZ* probe and high-molecular-weight DNA prepared from tail biopsies of each potential founder. The number of copies per cell was quantitated by comparing the hybridization signal intensity to standards corresponding to 1, 10, and 100 copies/cell with a Molecular Dynamics PhosphorImager. At least two independent founder lines containing each transgene were identified as described previously (52). Transgenic embryos (less than embryonic day [ED] 15.5) and tissue sections from adult mice were fixed, stained for β-galactosidase activity and counter-stained with hematoxylin and eosin as described previously (30). Of note, 0.02% Nonidet P-40 was added to phosphate-buffered saline during the fixation of whole mount embryos. In addition, to visualize the arterial system of mouse embryos after staining for β-galactosidase activity, embryos were dehydrated in methanol for 48 h and cleared in 2:1 (vol/vol) benzyl benzoate-benzyl alcohol for 2 h.

RESULTS

Analysis of the *cis*-acting elements that control the lineage-restricted pattern of SM22α transcription in transgenic mice. We have reported previously that the 441-bp SM22α promoter is sufficient to program high-level transcriptional activity in both primary rat aorta SMCs and the immortalized rat aorta SMC line A7r5 (63). To confirm that the 441-bp SM22α promoter was sufficient to activate transcription in a SMC lineage-restricted pattern in vivo, six independent lines of transgenic mice harboring a transgene containing the 441-bp SM22α promoter subcloned immediately 5′ of the bacterial β-galactosidase reporter gene (*lacZ*) were produced. The six founders contained between 5 and 160 copies per cell, as assessed by Southern blot analysis (Table 1, row 1). In ED 11.5 embryos, the endogenous SM22α gene is expressed throughout the primitive heart tube, developing somites, dorsal aorta, and the forming branch arteries (data not shown and reference 27). Whole mount staining of ED 11.5 embryos demonstrated high-level β-galactosidase activity throughout the developing arterial system (Fig. 1A). Blue staining was observed throughout the dorsal aorta, the carotid and vertebral arteries, the cerebral arteries, the umbilical arteries, and the aortic arches (Fig. 1A). A high-power section through the iliac artery, demonstrated that expression of the *lacZ* transgene was restricted to one to two layers of cells underlying the arterial endothelium (Fig. 1B). In addition, β-galactosidase activity was detected within the myotomal component of the developing somites (Fig. 1A and C) and within the bulbo-truncus region (future outflow tract) (Fig. 1D) and at low levels within the bulbo-cordis region (future right ventricle) (Fig. 1D) of the primitive heart. β-Galactosidase activity was not detected within the future left ventricle, left atrium (Fig. 1D), or right atrium (Fig. 1D) at this

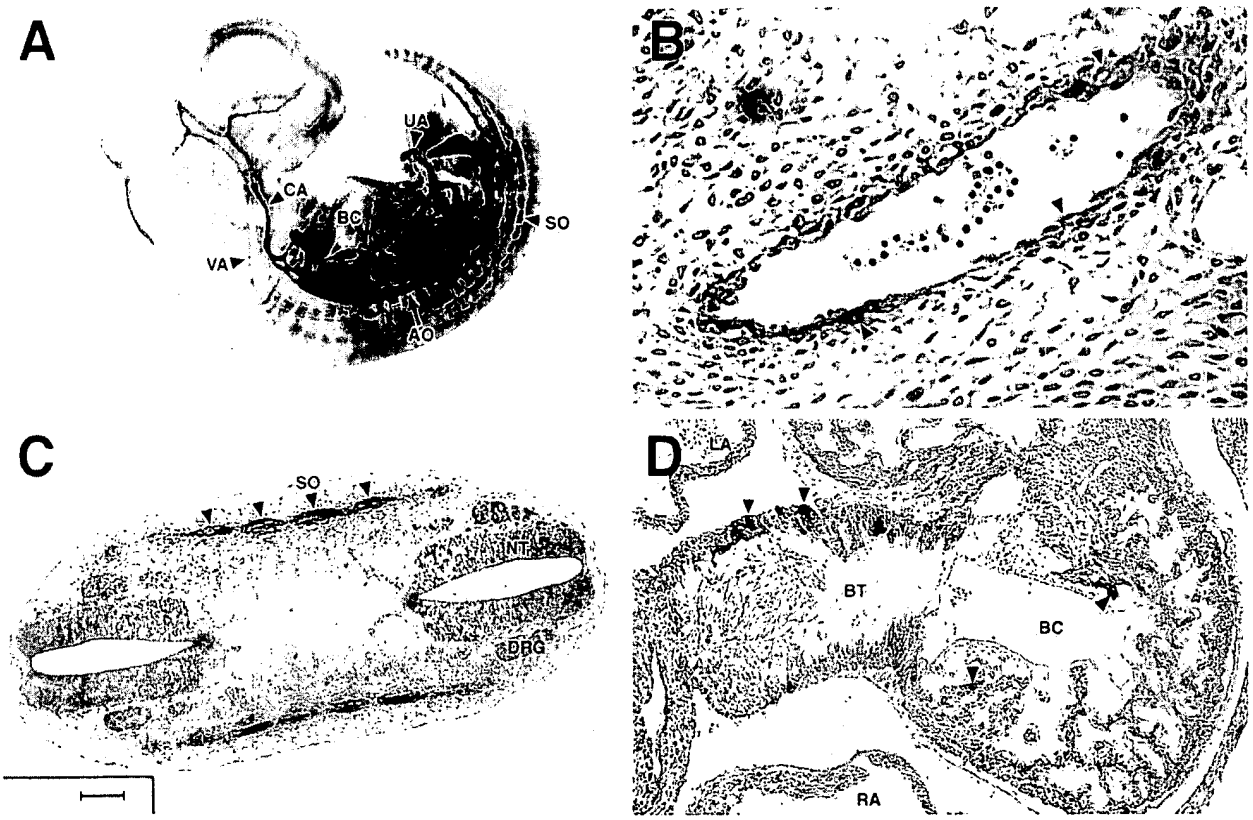

FIG. 1. The 441-bp SM22α promoter directs arterial SMC lineage-restricted gene expression in transgenic mice. (A) ED 11.5 transgenic embryos harboring the −441SM22-lacZ transgene were isolated, fixed, and stained for β-galactosidase activity. Expression of the *lacZ* reporter gene (blue staining) is evident in the dorsal aorta (AO), carotid arteries (CA), vertebral arteries (VA), umbilical artery (UA), and the smaller branch arteries. In addition, β-galactosidase activity was present in the cardiac outflow tract and the myotomal component of the somites (SO). (B) Transgenic embryos were sectioned and counterstained with hematoxylin and eosin. In this section through an embryonic artery, β-galactosidase activity is restricted to the layer of cells underlying the endothelium (arrows). Original magnification, ×40. (C) Coronal section demonstrating β-galactosidase activity in the myotomal component of the somites (SO). Original magnification, ×7.5. (D) Section at the level of the cardiac outflow tract demonstrating β-galactosidase activity in the SMCs of the bulbo-truncus (BT) region and within several cardiac myocytes in the bulbo-cordis (BC) region of the primitive heart. Original magnification, ×20.

stage of embryonic development. Surprisingly, although the SM22α gene is expressed at high levels in SMCs lining the pulmonary airways, as well as within the gastrointestinal and genitourinary tracts, no β-galactosidase activity was detected in the developing lung buds, gastrointestinal mucosa, or uterine or bladder mucosa during late murine embryogenesis or postnatal development (data not shown).

We have reported previously that truncation of the SM22α promoter from 441 to 300 bp reduces SM22α promoter activity by approximately 50% after transfection into cultured vascular SMCs (63). However, it is noteworthy that the normalized luciferase activity obtained with the 300-bp promoter was still approximately 500-fold above that obtained with promoterless control plasmids in these transient transfection assays. To determine whether a 280-bp SM22α promoter fragment (bp −280 to +41) was sufficient to direct arterial SMC-specific gene expression, we produced seven independent lines of transgenic mice in which the *lacZ* gene was placed under the transcriptional control of the 280-bp SM22α promoter. These mice contained between 2 and 34 copies of the transgene per cell (Table 1, row 2). As shown in Fig. 2A, 280-bp of 5′ flanking sequence was sufficient to direct high-level β-galactosidase activity (blue staining) to arterial SMCs (arrows) and the myotomal component of the somites of ED 11.5 mice. Of note, virtually identical patterns of transgene expression were demonstrated in four independent lines analyzed at ED 11.5. Dense blue staining was detected within the cardiac outflow tract (Fig. 2C), while a somewhat patchy pattern of blue staining was present in the developing arterial system (Fig. 2C). Higher-power sections confirmed that virtually every cell within the cardiac outflow tract stained blue (Fig. 2B). In addition, most, but not all, cells underlying the epithelium of the developing arteries stained blue (Fig. 2B). Interestingly, blue staining was detected within the mesenchymal cells that compose the aorticopulmonary spiral septum, which is present at ED 11.5 (Fig. 2D). Taken together, these data demonstrate that the 280-bp SM22α promoter is sufficient to program lineage-restricted transcription in arterial SMCs and the developing somites (Table 1, row 2). However, in contrast to the endogenous pattern of SM22α gene expression, the 441-bp (and 280-bp) SM22α promoter does not contain the *cis*-acting elements that control SM22α transcription in either visceral (gastrointestinal, uterine, bladder, and bronchial) or venous SMCs or in the primitive heart tube. Finally, it is noteworthy that we observed virtually the same arterial SMC-specific pattern of expression with the 5,000-bp SM22α promoter in transgenic mice (Table 1, row 3). These data strongly suggest that distinct transcriptional programs distinguish tissue-restricted subsets of SMCs (even within the vasculature).

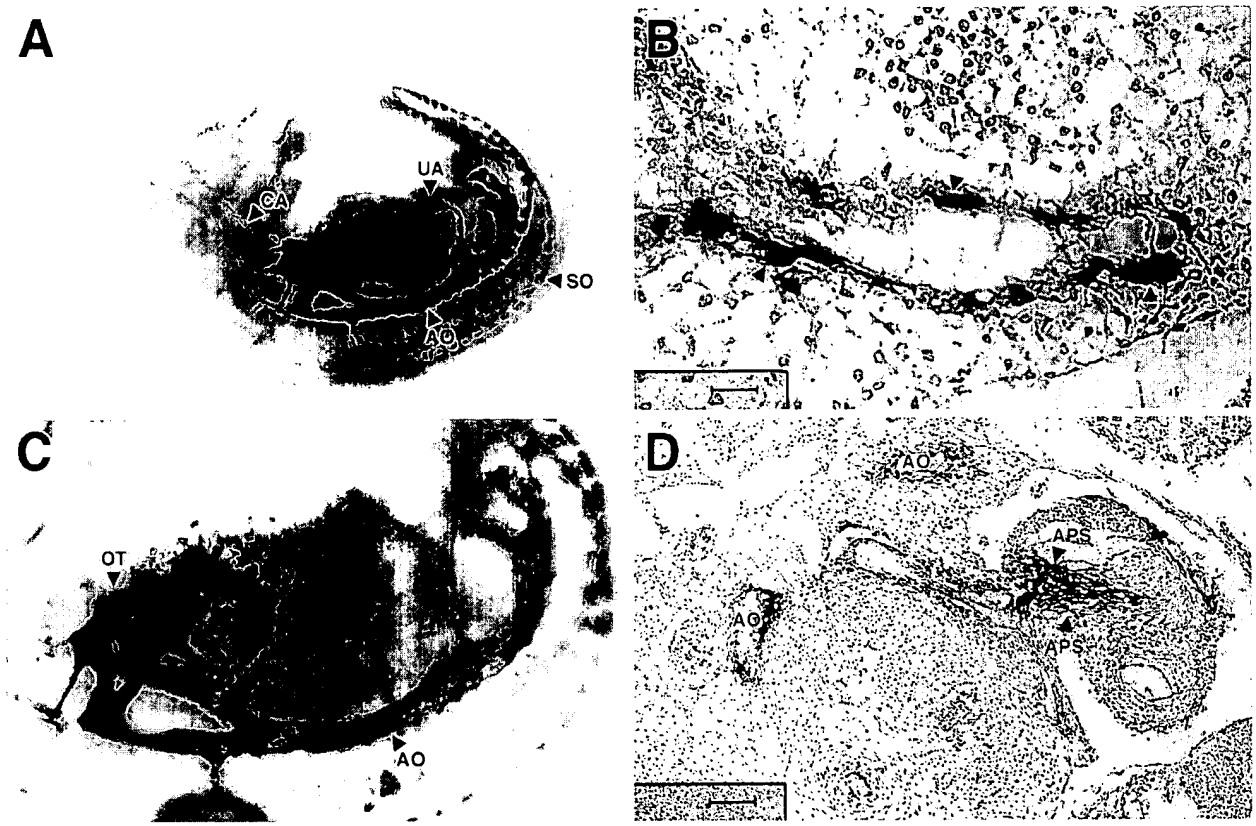

FIG. 2. The 280-bp SM22α promoter is sufficient to restrict expression of the *lacZ* reporter gene to the arterial SMCs and the somites in transgenic mice. (A) ED 11.5 transgenic mice harboring the −280SM22-lacZ transgene were isolated, fixed, and stained for β-galactosidase activity. Expression of the *lacZ* reporter gene (blue staining) is evident in the dorsal aorta (AO), carotid arteries (CA), vertebral arteries (VA), umbilical artery (UA), and the smaller branch arteries. In addition, β-galactosidase activity was present in the cardiac outflow tract and the myotomal component of the somites (SO). (B) Tangential section through the embryonic aorta demonstrating β-galactosidase activity in the cells underlying the endothelium (arrows). Bar, 50 μm. (C) Higher-power view of the differential pattern of staining in the cardiac outflow tract (OT) and dorsal aorta (AO). In contrast to the uniformly dark blue pattern of expression observed in the dorsal aorta and branch arteries of the −441SM22-lacZ transgenic mice (Fig. 1A), patchy blue staining was observed in the dorsal aorta and branch arteries, while dark blue staining was observed in the cardiac outflow tract (OT). (D) In this coronal section through the cardiac outflow tract, many blue-staining cells are seen within the mesenchymal tissue that makes up the aorticopulmonary spiral septum (APS). In addition, β-galactosidase activity was observed in the cells around the unfused right and left aorta (AO). Bar, 200 μm.

Identification of nuclear protein binding sites in the SM22α promoter. To identify nuclear protein binding sites within the 441-bp SM22α promoter, DNase I footprint analyses were performed with nuclear extracts prepared from the SMC line A7r5 (which express high levels of SM22α mRNA [63]), and the non-muscle cell line, NIH 3T3 (Fig. 3A to C). Six nuclear protein binding sites were identified on both the sense and antisense strands with nuclear extracts prepared from the SMC line A7r5. The six nuclear protein binding sites were designated SMEs −1 to −6, respectively (Fig. 3D). Two footprinted regions, SME-1 (bp −279 to −256) and SME-4 (bp −171 to −136), contain embedded SREs, or CArG boxes (CC WWWWWWGG) (Fig. 3D), that have been shown previously to bind the MADS box transcription factor SRF and that play an important role in regulating transcription of the genes encoding skeletal and cardiac α-actin (36, 38, 40). Of note, fine differences in the digestion patterns between nuclear extracts prepared from A7r5 and NIH 3T3 cells could be distinguished over the SME-4 site (Fig. 3B, compare lanes 4 and 5 and 6 and 7; Fig. 3B, compare lanes 11 and 12 and 13 and 14). Several studies suggest that nucleotides embedded within and/or flanking CArG boxes regulate binding of ternary complex factors, including members of the *ets* and homeodomain families of transcription factors. Thus, the finding of consensus *ets* binding sites (AGGA) 3 bp (on the antisense strand) and 22 bp (on the sense strand), respectively, 5′ of the CArG box embedded within the SME-1 motif is noteworthy. Similarly, SME-4 spans a GGAG motif (bp −142 to −139) which has been demonstrated to bind to ternary complex factors in the *ets* family of transcription factors (20). Moreover, the SME-4 nuclear protein binding site contains the embedded motif ATATGG (bp −146 to −141), which has been demonstrated to bind homeobox transcription factors including Csx/Nkx2.5 (5).

The SME-2 nuclear protein binding site (bp −249 and −216) contains consensus binding motifs for the ubiquitously expressed transcription factors Sp1 (KRGGCKRRK) and AP2 (CCCMNSSS) (Fig. 3D, SME-2). The SME-3 nuclear protein binding site (bp −215 to −186), which is flanked by DNase I hypersensitive sites at both its 5′ and 3′ borders, was protected only by nuclear extracts prepared from A7r5 and not by extracts prepared from NIH 3T3 cells (Fig. 3B, compare lanes 4 and 5 and 6 and 7; Fig. 3B, compare lanes 11 and 12 and 13 and 14). This nuclear protein binding site has not been described previously. The SME-5 nuclear protein binding site (bp −86 to −66) once again contains consensus Sp1 and AP2 motifs (Fig. 3C). The SME-6 nuclear protein binding site (bp −59 to −35),

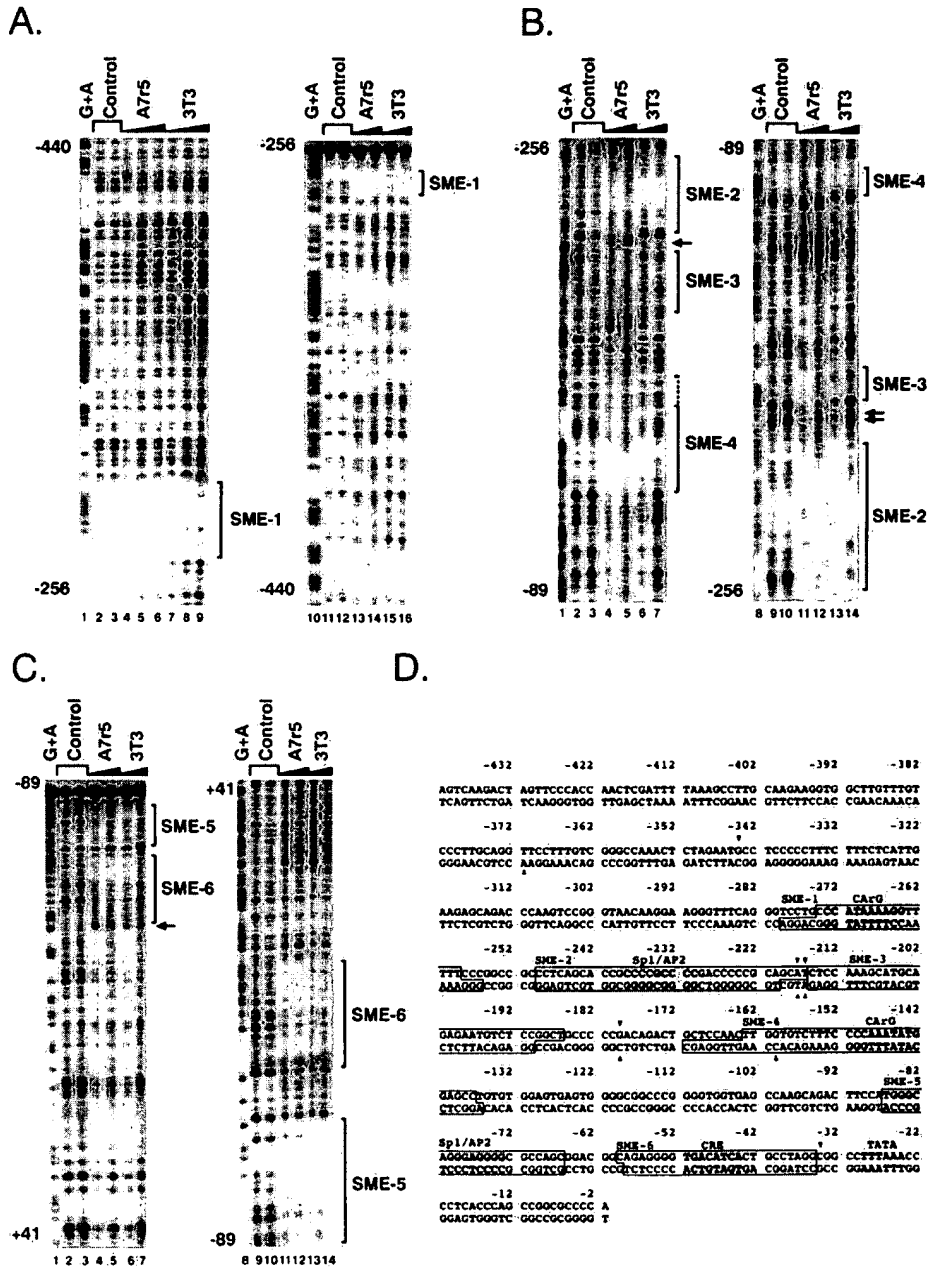

FIG. 3. DNase I footprint analysis of the SM22α arterial SMC-specific promoter. (A to C) Footprint analysis. Three genomic subfragments (bp −441 to −256 [A], bp −256 to −89 [B], and bp −89 to +41 [C]) spanning the 482-bp (bp −441 to +42) SM22α promoter were subjected to DNase I footprint analyses with nuclear extracts prepared from the SMC line A7r5 (which expresses high levels of SM22α mRNA) and NIH 3T3 cells. The sense (left panel) and antisense (right panel) strands of the three genomic subfragments were end labeled and incubated in the absence (control) or presence of A7r5 and NIH 3T3 (3T3) nuclear extracts before partial digestion with DNase I (concentrations varied from 5 to 22.5 U/ml). Standard Maxam and Gilbert purine (G + A) sequencing reactions were run in parallel. The six protected regions identified on both strands with A7r5 nuclear extracts were designated SME-1 to -6, respectively, and are bracketed. DNase I hypersensitive sites are indicated with arrows. (D) Nucleotide sequence of the 441-bp SM22α arterial SMC-specific promoter. The six nuclear protein binding sites identified with A7r5 nuclear extracts are boxed. DNase I hypersensitive sites are indicated by arrowheads. Consensus transcription factor binding motifs are shaded gray and denoted above the nucleotide sequence.

lies immediately 5′ of the nonconsensus TATA box (TTTAA), and contains nucleotide sequences that have been demonstrated previously to bind the cyclic AMP response element (CRE) binding proteins (for review, see reference 24) (Fig. 3C). Of note, an AT-rich sequence (bp −408 to −415) with 8 of 10 bp of sequence identity with the consensus MEF2 binding motif (15) was not protected with either A7r5 or NIH 3T3 nuclear extracts. In summary, these experiments demonstrated six nuclear protein binding sites within the murine SM22α promoter (Fig. 3D). One of these binding sites (SME-3) demonstrated differential patterns of digestion when incubated with nuclear extracts prepared from A7r5 and NIH 3T3 cells.

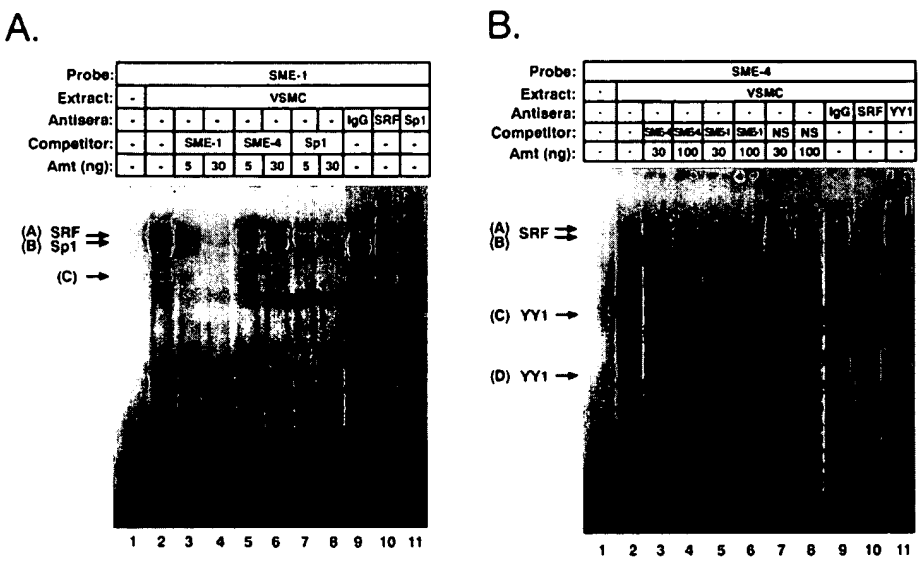

FIG. 4. EMSAs of the SME-1 and SME-4 nuclear protein binding sites of the arterial SMC-specific SM22α promoter. (A) Identification of nuclear protein complexes that bind to SME-1. Radiolabeled oligonucleotides corresponding to the SME-1 binding site were subjected to EMSAs with 10 μg of nuclear extracts prepared from primary rat aortic SMCs (VSMCs). Some binding reaction mixtures included 5 to 30 ng of the indicated unlabeled competitor oligonucleotides or 1 μl of the indicated antiserum. Three specific complexes were detected and are designated A, B, and C to the left of the autoradiogram. Nuclear protein complexes which were ablated and supershifted by α-SRF and α-Sp1 antiserum are indicated with arrows to the left of the autoradiogram. (B) Identification of nuclear protein complexes which bind to SME-4. EMSAs were performed with a radiolabeled SME-4 oligonucleotide probe as described above. Four specific nuclear protein complexes were detected and are designated A to D to the left of the autoradiogram. Nuclear protein complexes which were ablated and supershifted by α-SRF and α-YY1 antisera are indicated with arrows to the left of the autoradiogram.

In addition, fine differences in the patterns of digestion with A7r5 and NIH 3T3 cell extracts were observed over the SME-4 nuclear protein binding site that contains an embedded CArG box.

Characterization of *trans*-acting factors that bind to the SM22α promoter. To assess the number, specificity, and identity of nuclear proteins that bind to the two CArG box-containing elements, SME-1 and SME-4, as well as the SME-3 site, which was differentially footprinted with SMC and non-SMC nuclear extracts, a series of EMSAs were performed. The radiolabeled SME-1 oligonucleotide probe bound three specific nuclear protein complexes, designated A to C (Fig. 4A, lane 2), as determined by addition of specific and nonspecific unlabeled competitor oligonucleotides to the binding reaction mixtures (Fig. 4A, lanes 3 and 4 and data not shown). Of note, unlabeled SME-4 oligonucleotide competed for binding of complex A, but failed to compete for complexes B and C (Fig. 4A, lanes 5 and 6). Unlabeled Sp1 oligonucleotide competed for binding of complex B (that comigrated with complex A), as well as, complex C (Fig. 4A, lanes 7 and 8). Antibody supershift experiments confirmed that complex A (arrow) contains SRF (or an antigenically related protein) and complex B (arrow) contains Sp1 (or an antigenically related protein) (Fig. 4A, lanes 9 to 11).

EMSAs performed with the radiolabeled SME-4 oligonucleotide probe demonstrated four specific nuclear protein complexes, designated A to D (Fig. 4B, lane 2), as determined by addition of specific and nonspecific competitor oligonucleotides (lanes 3 and 4 and 7 and 8). Addition of unlabeled SME-1 oligonucleotide competed only for binding of complexes A and B (Fig. 4B, lanes 5 and 6). Antibody supershift experiments revealed that both of these low-mobility nuclear protein complexes contained a protein identical to or antigenically related to SRF (Fig. 4B, lane 10), while complexes C and D contained a protein identical to or antigenically related to YY1 (Fig. 4B, lane 11). Taken together, these data demonstrate that as expected, SRF (or an SRF-containing protein complex) binds to both the SME-1 and SME-4 sites. Of note, the demonstration of two low-mobility SME-4 binding activities containing SRF (Fig. 4B, complexes A and B) suggests that one, or both, of these complexes may contain additional *trans*-acting factors, or, alternatively, these complexes may represent an SRF doublet. In addition, SME-1 bound Sp1 (Fig. 4A, complex B) and one potentially novel nuclear protein complex (Fig. 4A, complex C) that does not bind to SME-4. Conversely, SME-4 binds the ubiquitously expressed and potentially negative regulatory factor YY1 (17, 25, 26) (Fig. 4B, complexes C and D), while SME-1 does not.

As discussed above, SME-3 was protected from DNase I digestion by nuclear extracts prepared from A7r5 cells, but not by extracts prepared from NIH 3T3 cells, suggesting that this previously undescribed motif might bind one or more SMC lineage-specific *trans*-acting factors. EMSAs performed with the radiolabeled SME-3 oligonucleotide probe revealed three specific binding activities, designated A to C (Fig. 5, lane 2), as determined by addition of specific and nonspecific competitor oligonucleotides (Fig. 5, lanes 3 to 6). Antibody supershift experiments revealed that complexes B and C contained YY1 (or an antigenically related protein) (Fig. 5, lane 9). None of the nuclear protein complexes were supershifted by control IgG- or α-Sp1-specific antiserum (Fig. 5, lanes 7 and 8). To determine whether any of these nuclear protein complexes were expressed in a lineage-restricted fashion, EMSAs were performed with the radiolabeled SME-3 probe and nuclear extracts prepared from primary rat aortic SMCs, the immortalized SMC line A7r5, C3H10T1/2 and NIH 3T3 fibroblasts, and the mouse T-cell line EL4. Interestingly, complex C, which was ablated by preincubation with α-YY1 antiserum, was present only in primary rat aortic SMCs and the SMC line A7r5 (Fig. 5, lane 2 and 10). Conversely, three faint complexes

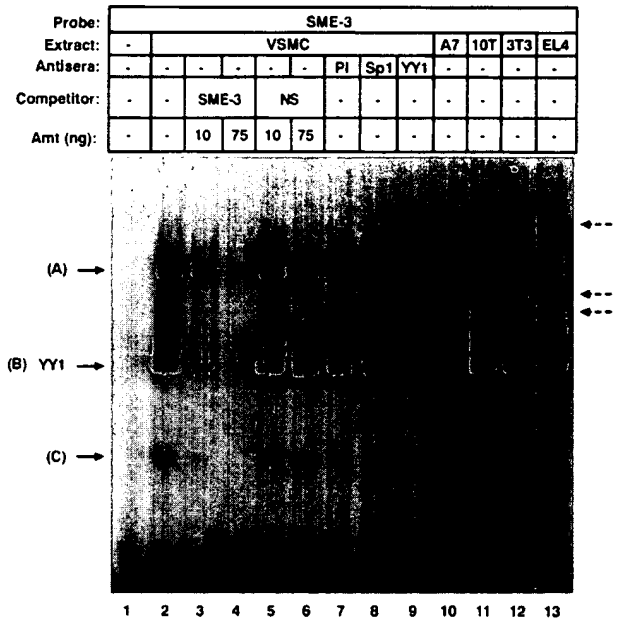

FIG. 5. EMSAs of the SME-3 nuclear protein binding site of the SM22α promoter. EMSA performed with the radiolabeled SME-3 oligonucleotide probe and nuclear extracts prepared from primary rat aortic SMCs (VSMC) or A7r5 (A7) C3H10T1/2 (10T), NIH 3T3 (3T3), and EL4 cells. Some binding reaction mixtures included between 10 and 75 ng of the indicated unlabeled competitor oligonucleotides. Three specific binding activities, designated A to C, were identified as denoted to the left of the autoradiogram. Nuclear protein complexes that were ablated and supershifted by α-YY1-specific antisera are indicated with arrows to the left of the EMSA. Of note, complex C was present only in nuclear extracts prepared from SMC lineages (arrow). In addition, three unique nuclear protein complexes were present in non-SMC nuclear extracts but not in SMC extracts (dashed arrows).

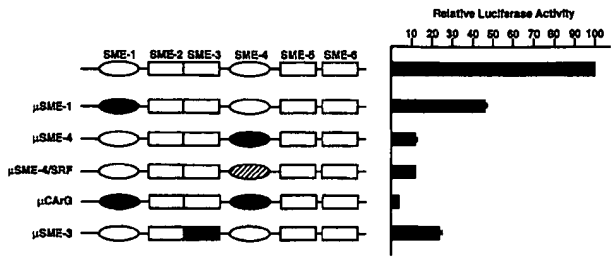

FIG. 6. Mutational analyses of the SME-1, SME-3, and SME-4 nuclear protein binding sites on SM22α promoter activity in arterial SMCs. Mutations were introduced into SME-1, SME-3, and SME-4 within the context of the 441-bp SM22α promoter as described in Materials and Methods. The mutant promoters were subcloned into the pGL2-Basic luciferase reporter plasmid, and the resulting plasmids were transfected into primary rat aortic SMCs. A schematic representation of the SM22α promoter and of the mutated *cis*-acting elements (indicated by black) is shown to the left of the graph. In addition, a mutation that selectively abolished binding of SRF, but not YY1, to SME-4 is indicated by a hatched oval. Luciferase activities, corrected for differences in transfection efficiencies, are shown as percentage of the luciferase activity observed with the p-441SM22luc plasmid ± standard error. Each transfection was repeated at least three times.

that were not present in SMC extracts were identified in C3H10T1/2, NIH 3T3, and EL4 cells (Fig. 5, lanes 11 to 13). Taken together, these data suggest that the SME-3 nuclear protein binding site, a motif which has not been described previously, binds YY1 and one or more, as yet, unidentified SMC-specific and/or lineage-restricted *trans*-acting factors. In addition, the radiolabeled SME-3 probe binds three nuclear protein complexes that are present in several non-SMC lines, but not in primary vascular SMCs or the SMC line A7r5.

Mutational analysis of the SM22α promoter. To determine the functional significance of the SME-1 and SME-4 sites, each of which binds SRF (as well as several distinct *trans*-acting factors), specific mutations that abolish binding of one or more of the nuclear protein complexes to SME-1 and/or SME-4 (as determined by EMSAs) were introduced into the SM22α promoter. The functional effect of each mutation was assessed by transient transfection analysis of primary rat aortic SMCs with each SM22α promoter mutant luciferase reporter plasmid. A mutation in the SME-1 site that completely abolished binding of SRF and Sp1 (complexes A to C in Fig. 4A) resulted in a 55% reduction in normalized luciferase activity compared to that obtained with the p-441SM22luc plasmid (Fig. 6, lanes 1 and 2). In contrast to this relatively modest reduction in luciferase activity, a mutation in the SME-4 site that abolished binding of both SRF and YY1 (complexes A to D in Fig. 4B) resulted in an 88% reduction in normalized luciferase activity compared to that obtained with the wild-type SM22α promoter (Fig. 6, lane 3). To determine whether this decrease in transcriptional activity was due to binding of SRF (and SRF cofactors) to SME-4, a dinucleotide substitution that eliminates binding of SRF (complexes A and B in Fig. 4B), but not YY1 (complexes C and D in Fig. 4B), to SME-4 was introduced in the 441-bp SM22α promoter. Transfection of the luciferase reporter plasmid containing this mutation, p-441SM22μSME-4/SRF, resulted in an 88% reduction in normalized luciferase activity (Fig. 6, lane 4). Finally, the p-441SM22μCArG plasmid, containing mutations in both SME-1 and -4 that eliminate SRF binding activities, completely abolished transcriptional activity of the SM22α promoter in primary rat aorta SMCs and the SMC line A7r5 (Fig. 6, lane 5, and data not shown).

Previous studies have demonstrated that CArG motifs regulate skeletal muscle cell-specific transcription in concert with other transcriptional regulatory elements (often located in close physical proximity) that bind lineage-specific transcription factors (59). To determine the functional significance of the previously undescribed SME-3 nuclear protein binding site, which lies adjacent to the SME-4/CArG motif, a mutation in SME-3 was introduced into the 441-bp SM22α promoter that eliminated each SME-3 binding activity (complexes A to C in Fig. 5). Transfection of the p-441SM22μSME-3luc reporter plasmid into primary aorta SMCs resulted in an 80% reduction in normalized luciferase activity compared to that obtained with the native SM22α promoter containing the luciferase reporter plasmid p-441SM22luc (Fig. 6, lane 6). Taken together, these data demonstrate that mutations in SME-3 or mutations that abolish binding of SRF (and factors that bind directly to SRF) to SME-4 are required for high-level activity of the SM22α promoter in arterial SMCs in vitro.

YY1-modulated activation of the SM22α promoter in primary vascular SMCs. The mutational analyses described above suggested that SME-4 plays a critical role in regulating activity of the SM22α promoter in arterial SMCs (Fig. 6, lanes 3 and 4). In addition, as shown in Fig. 4B, EMSAs revealed that both SRF and YY1 bind to SME-4. Of note, two additional YY1-binding sites were identified in the SM22α promoter, SME-3 and SME-6 (Fig. 5 and data not shown). YY1 is a multifunctional transcription factor that has the capacity to bend DNA and that functions as a transcriptional activator or repressor in different contexts (17, 25, 42, 43). To determine whether YY1 activated or repressed transcription from the SM22α promoter in arterial SMCs, and which of the three

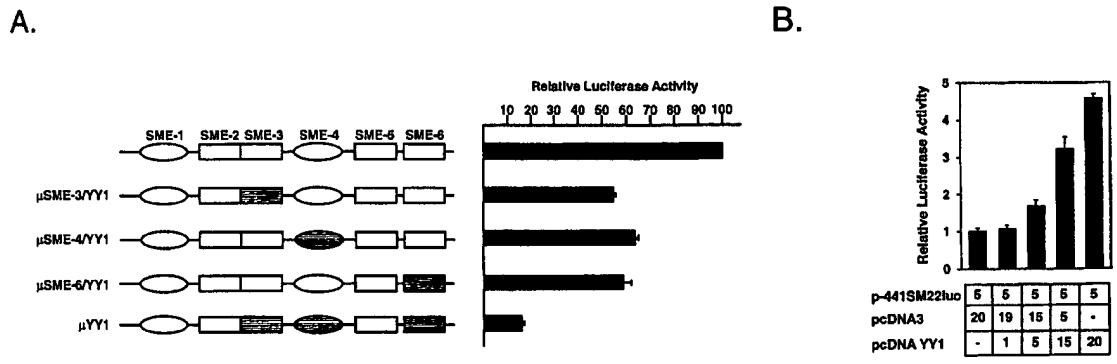

FIG. 7. YY1-modulated activation of the SM22α promoter in SMCs. (A) Mutational analysis of the SM22α promoter. Mutations that selectively abolish binding of YY1 to SME-3, SME-4, and SME-6 and mutations that abolish binding of YY1 to each respective site were introduced into the 441-bp SM22α promoter. The mutant promoters were subcloned into the pGL2-Basic luciferase reporter plasmid, and the resulting plasmids were transfected into primary rat aortic SMCs. A schematic representation of the SM22α promoter and of the mutated *cis*-acting elements (indicated by horizontal stripes) is shown to the left of the graph. Luciferase activities, corrected for differences in transfection efficiencies, are shown as a percentage of the luciferase activity observed with the p-441SM22luc plasmid ± standard error. (B) Effect of overexpression of YY1 on SM22α promoter activity in SMCs. Primary rat aortic SMCs were transfected with 0 to 20 μg of the pcDNAYY1 expression plasmid encoding YY1 or the control expression plasmid pcDNA3, 5 μg of the p-441SM22luc reporter plasmid, and 5 μg of the pMSVβgal reference plasmid. The amount (micrograms) of plasmid transfected is shown in the boxes to the right of each plasmid. Forty-eight hours after transfection, luciferase activity (in light units) and β-galactosidase activities were determined from cell lysates. Relative luciferase activity was determined by correcting for differences in transfection efficiencies and normalizing the light units obtained after cotransfection of the p-441SM22luc reporter plasmid with the pcDNA3-negative control expression plasmid. The data are presented as relative luciferase activity ± standard error.

YY1-binding sites mediated these effects, selective mutations that abolished binding of YY1 to the SME-3, -4, and -6 sites, respectively, and YY1 to all three sites were created within the context of the native 441-bp SM22α promoter. A mutation that abolished binding of YY1 to SME-3 (complexes B and C in Fig. 5) caused a 45% reduction in transcriptional activity compared to that obtained with the p-441SM22luc reporter plasmid (Fig. 7A, lanes 1 and 2). Similarly, a mutation that abolished binding of YY1 to SME-4 (complexes C and D in Fig. 4B) caused a 35% reduction in normalized luciferase activity compared to that obtained with the native SM22α promoter (Fig. 7A, lane 3). In addition, a mutation that abolished binding of YY1 to SME-6 (data not shown) caused a 40% reduction in normalized luciferase activity compared to that obtained with the p-441SM22luc control plasmid (Fig. 7A, lane 4). In contrast to the relatively modest reductions in luciferase activity observed with selective mutations of SME-3, -4, and -6, an 80% reduction in normalized luciferase activity was demonstrated when all three YY1-binding sites were mutated in the context of the 441-bp SM22α promoter (Fig. 7A, lane 5).

In order to examine whether overexpression of YY1 would activate or repress activity of the SM22α promoter in vascular SMCs, primary rat aortic SMCs were transiently cotransfected with an eukaryotic expression plasmid encoding the human YY1 protein and the p-441SM22luc reporter plasmid. Consistent with our previous studies (63), the p-441SM22luc reporter plasmid increased luciferase activity 2,500-fold above levels obtained with the promoterless control luciferase reporter plasmid pGL2-Basic, in primary vascular SMCs, a value set to 1 in these analyses (Fig. 7B, lane 1). Cotransfection of the p-441SM22luc reporter plasmid with increasing amounts of the YY1 expression plasmid (from 0 to 20 μg) resulted in stepwise increases in the normalized luciferase activities (up to 450%) above levels obtained with the p-441SM22luc reporter plasmid (Fig. 7B, lanes 2 to 5). Taken together, these data demonstrate that YY1 activates transcription from the SM22α promoter in arterial SMCs.

Functional characterization of the SM22α promoter in transgenic mice. In order to confirm the functional importance of the two elements that bind SRF (SME-1 and SME-4) and the previously undescribed SME-3 nuclear protein binding site in vivo, transgenic mice were produced that harbor transgenes encoding the bacterial *lacZ* reporter gene under the transcriptional control of the −441SM22μCArG and −441SM22μSME-3 promoter mutants, respectively. Twelve independent −441SM22 μCArG transgenic lines were produced with copy numbers ranging between 1 and 730 copies per cell (Table 1, row 4). In contrast to the −441SM22lacZ transgenic mice that expressed the *lacZ* transgene in the arterial SMCs and within the myotomal component of the somites (Fig. 1A), in 11 of 12 independent −441SM22μCArG lines, β-galactosidase activity could not be detected in either the arterial SMCs or within the myotomal component of the somites at ED 11.5 (Table 1, row 4). Of note, in one transgenic line harboring the −441SM22μCArG transgene (which contained five copies per cell), blue staining was detected exclusively within the cardiac outflow tract, but not within the SMCs of the dorsal aorta or branch arteries, the somites, or any other tissue. Given the low frequency at which this pattern of *lacZ* expression was observed, it is likely that it resulted from integration of the transgene near a cryptic enhancer element. In addition, two independent lines of transgenic mice were produced which contained two and five copies per cell, respectively, of the −441SM22μSME-3 transgene (Table 1, row 5). Once again, β-galactosidase activity was not detected within the arteries or somites of either line of p-441SM22μSME-3 transgenic mice (Table 1, row 5). Taken together, these data demonstrate that mutations in the SME-1 and SME-4 nuclear protein binding sites which eliminate binding of SRF to the SM22α promoter, or a mutation of the SME-3 site which eliminates each SME-3 binding activity, abolish activity of the SM22α promoter in arterial SMCs and within the myotomal component of the somites in vivo. These data suggest that combinatorial interactions between *trans*-acting factors that bind to the two CArG elements, SME-1 and SME-4, as well as factors that bind to SME-3, are required for activity of the SM22α promoter in vivo.

The SME-4 element is necessary and sufficient to direct high-level SMC lineage-restricted transcription in vitro. The mutational analyses described above suggested strongly that SME-4 plays a critical role in regulating activity of the SM22α

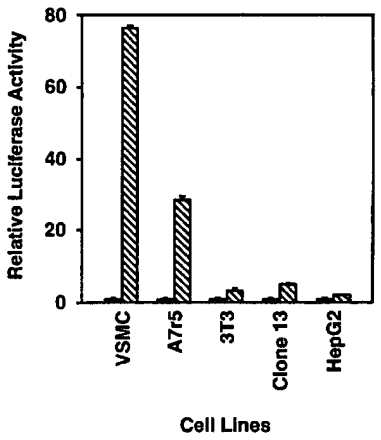

FIG. 8. Cellular specificity of a multimerized SME-4 element. Four copies of a double-stranded oligonucleotide corresponding to the SME-4 motif (bp −190 to −110) were subcloned immediately 5′ of a minimal SM22α promoter (bp −90 to +41) in a luciferase reporter plasmid, designated pSME-4X4luc. The pSME-4X4luc reporter plasmid (hatched bars) and the p-90SM22luc reporter plasmid (solid bars) were transiently transfected into primary rat aortic SMCs (VSMCs), the SMC line A7r5, and three non-SMC lines (NIH 3T3, clone 13, and HepG2), and the normalized luciferase activities for each respective plasmid were determined as described in Materials and Methods. These data are presented as relative luciferase activity ± standard error.

promoter in arterial SMCs. To examine whether this element is sufficient to regulate the cell specificity of the SM22α promoter, four copies of the SME-4 element (and 18 bp of flanking sequence) were subcloned immediately 5′ of a minimal SM22α promoter (bp −90 to +41) in a luciferase reporter plasmid designated p-90SME-4X4luc. Transfection of primary rat aorta SMCs with the p-90SME-4X4luc plasmid resulted in an 80-fold induction in normalized luciferase activity compared to that obtained with the p-90SM22luc plasmid containing the minimal SM22α promoter (Fig. 8, lane 1). Similarly, in the rat aortic SMC line A7r5, a 30-fold induction in promoter activity was demonstrated (Fig. 8, lane 2). In contrast, only 5-fold, 2-fold, and 3-fold inductions in normalized luciferase activities were obtained with the p-90SME-4X4luc reporter plasmid after transient transfection of the non-SMC lines NIH 3T3, clone 13, and HepG2, respectively (Fig. 8, lanes 3 to 5). These data demonstrate that a multimerized copy of SME-4 (bp −190 to −110) when linked to the minimal SM22α promoter is necessary and sufficient to direct high-level transcription in an SMC lineage-restricted fashion.

DISCUSSION

Relatively little is currently understood about the molecular mechanisms that control SMC-specific gene expression and those which underlie SMC diversity. In the studies described in this report, the SM22α promoter was used as a model system for identifying the *cis*-acting elements and *trans*-acting factors that control SM22α gene expression in SMCs. These studies revealed that despite the fact that SM22α gene is expressed in both vascular and visceral SMCs in vivo (10, 12, 44, 63), the SM22α promoters activate transcription exclusively in arterial SMCs, but not in visceral and venous SMCs (Table 1). The 280-bp arterial SMC-specific SM22α promoter contains six nuclear protein binding sites, designated SME-1 to -6, respectively, that bind multiple transcription factors, including SRF, Sp1, and YY1, as well as several potentially novel *trans*-acting factors expressed in arterial SMCs. Functional analyses of the SM22α promoter revealed that mutations that eliminate binding of SRF to SME-1 and SME-4 or mutations of SME-3 abolish activity of the SM22α promoter in arterial SMCs and in the myotomal component of the somites in transgenic mice. Moreover, a multimerized SME-4 element, in conjunction with the minimal SM22α promoter, is sufficient to program SMC lineage-restricted gene expression in vitro. Taken together, functional characterization of the 280-bp arterial SMC-specific promoter suggests a model wherein combinatorial interactions between SRF and other novel SMC lineage-specific transcription factors regulate SM22α gene expression and modulate the SMC phenotype.

During postnatal development, the SM22α gene is expressed exclusively in vascular and visceral SMCs (10, 12, 44, 63). In addition, recent studies have demonstrated that like other SMC-specific markers, including SM–α-actin and calponin, the SM22α gene is transiently expressed in the embryonic heart and somites (2, 33, 57, 58). We reported previously that 441 bp of 5′ flanking sequence is sufficient for high-level tissue-specific transcription of the SM22α gene in primary rat aortic SMCs and the rat aortic SMC line A7r5, but that this transcriptional regulatory element was inactive in non-muscle cell lineages (63). Thus, we anticipated that histochemical analysis of the −441SM22-lacZ transgenic mice would reveal β-galactosidase activity within both the vascular and visceral SMCs within the developing mouse; a pattern consistent with the pattern of expression of the endogenous SM22α gene in vivo. Surprisingly, β-galactosidase activity was detected exclusively in arterial, but not in visceral or venous, SMCs. These data are in agreement with the recent study of Li et al. (28), who also observed that the SM22α promoter is active exclusively in arterial SMCs during postnatal development. Moreover, the present studies extend the previous analyses and reveal that 280 bp of 5′ flanking sequence, containing six nuclear protein binding sites and two CArG motifs, is sufficient to restrict expression of a *lacZ* transgene to arterial SMCs. Thus, the *cis*-acting elements and *trans*-acting factors that control expression of the SM22α promoter in arterial SMCs may represent a "default" transcriptional regulatory program for the SMC lineage(s) that requires additional *cis*-acting elements (and *trans*-acting factors) to activate transcription in visceral and venous SMCs. The identification of the *cis*-acting sequences and *trans*-acting factors that control transcription of the SM22α gene in visceral SMCs should provide important insights into the transcriptional programs that underlie SMC diversity and the transcriptional targets that distinguish these SMC sublineages.

Although members of the MADS box family of transcription factors, including SRF and MEF2, have been implicated in regulating SMC-specific gene expression (2, 21, 29), the molecular mechanisms underlying their capacity to activate SMC-specific gene expression remain to be elucidated. The demonstration that (i) mutations in the two elements that contain embedded CArG boxes, SME-1 and SME-4, abolish SM22α promoter activity in the arterial SMCs of transgenic mice, that (ii) a two-nucleotide substitution within the SME-4 motif that selectively abolishes binding of SRF severely attenuates SM22α promoter activity in arterial SMCs, and that (iii) a multimerized oligonucleotide corresponding to SME-4 (and 18 bp of flanking sequence) is sufficient to activate transcription in arterial SMCs (and in A7r5 cells), but is relatively inactive in non-SMC lines, suggests strongly that factors which bind to the SME-4 element and/or directly to SRF may be necessary and sufficient to activate transcription in an SMC lineage-restricted fashion.

Despite the finding that SREs, or CArG motifs, are present in transcriptional regulatory elements that control the expres-

sion of genes encoding SMC-specific proteins (2, 3, 21, 68), it remains unclear how SRF, which is expressed ubiquitously at the mRNA level (for review, see references 20 and 67), can by itself regulate arterial SMC-specific transcription. Interestingly, recent studies have revealed that during embryonic avian development, the SRF protein is expressed almost exclusively in developing smooth and striated muscle (7). However, we have recently observed that forced expression of SRF alone is not sufficient to activate the murine SM22α promoter in non-SMCs (21a). Thus, these data suggest a model wherein SRF and one or more potentially novel (SMC lineage-specific) *trans*-acting factors that bind to SME-4 and/or directly to SRF are required for high-level expression of the SM22α gene in arterial SMCs. Thus, it is noteworthy that the SME-4/CArG element contains an embedded homeodomain protein binding site (5) and an *ets* protein consensus binding site. As such, physical association of SRF with members of the *ets* and/or homeodomain families of transcription factors, which have been demonstrated previously to synergistically activate transcription of genes expressed in non-SMCs (6, 8, 16, 32), may play important roles in programming arterial SMC-specific gene expression. Alternatively, the demonstration that mutations that eliminate each of the SME-3 binding activities in the context of the native SM22α promoter also abolished SM22α promoter activity in arterial SMCs (and the somites) of transgenic mice suggests that combinatorial interactions between one or more of these factors and SRF may underlie the transcriptional program that controls gene expression in arterial SMCs. Finally, it is possible that negative regulatory factors could bind to the SM22α promoter and restrict the cell specificity of its activity. However, mutations in each of the six SMEs within the context of the 441-bp SM22α promoter fail to activate transcription from the SM22α promoter in non-SMC lines (21a). Thus, these studies serve to identify SME-4 and proteins that bind to and functionally synergize with SME-4 and/or SRF as important regulators of arterial SMC-specific gene expression.

The molecular mechanisms underlying the capacity of arterial SMCs to reversibly modulate their phenotype in response to vascular injury are poorly understood (13, 39, 49, 62). Histopathological studies have revealed that while high levels of SM22α mRNA are present in arterial SMCs located within the tunica media, SM22α mRNA cannot be detected in synthetic SMCs that are present in atherosclerotic plaques (61). This suggested that both positive and negative regulatory mechanisms might control expression of the SM22α gene in arterial SMCs. Elucidation of the *trans*-acting factors that bind to the SM22α promoter has served to identify several candidates that might suppress transcription of the SM22α gene (and modulate the arterial SMC phenotype). The critically important SME-4 element binds not only SRF, but also YY1, a ubiquitously expressed transcription factor that can bind to CArG motifs in such a way as to preclude SRF binding and antagonize SRF activity (17, 25, 26). Thus, we hypothesized that the SME-4 site could serve as a nodal point for both positive and negative regulation of the SM22α gene. However, mutations that selectively abolish binding of YY1 to SME-4 or mutations that abolish binding of YY1 to the SM22α promoter (by selective mutations within the SME-3, SME-4, and SME-6 sites) decreased SM22α promoter activity in vitro, suggesting that in the context of the SM22α promoter, YY1 functions as a transcriptional activator. Furthermore, overexpression of YY1 in primary vascular SMCs transactivated the SM22α promoter in vascular SMCs. In this regard, it is noteworthy that YY1 has been shown to facilitate the association of SRF to the SRE/CArG motif in the c-*fos* promoter, and together these factors synergistically activate transcription in some contexts (42). Alternatively, the demonstration that members of the CREB/ATF family bind to the SME-6 site in the SM22α promoter (21a) is consistent with the hypothesis that the induction of repressive factors, such as CREM or CREB-2 (that bind to CREs), may play a role in suppressing expression of the SM22α gene in response to specific stimuli. Thus, it will be interesting to examine whether these negative regulatory factors that potentially link events at the cell surface to the nucleus can repress SM22α promoter activity in arterial SMCs in response to vascular injury.

While these studies focused primarily on defining the *cis*-acting elements and *trans*-acting factors that control SMC-specific gene expression, it is also noteworthy that the 280-bp SM22α promoter was sufficient to program developmentally regulated, lineage-restricted transcription in the myotomal component of the somites. Moreover, mutations that abolished binding of SRF to the SM22α promoter (and arterial SMC-specific gene expression) also abolished transgene expression in the developing somites. These data suggest that an SRF-dependent transcriptional program may also regulate somite-specific gene expression. Interestingly, the 441-bp SM22α promoter does not contain a consensus E box binding site for the bHLH family of myogenic transcription factors (for review, see reference 65). We have reported previously that the slow/cardiac troponin C (cTnC) gene, which is also transiently expressed in embryonic skeletal muscle, contains a transcriptional enhancer that lacks a binding site for bHLH transcription factors and is indirectly activated by bHLH family members (51). Thus, it will be interesting to determine whether bHLH family members are required for the functional activity of the SM22α promoter in the developing somites. Moreover, it will be important to determine whether distinct *trans*-acting factors regulate the activity of the SM22α promoter in arterial SMCs and within the myotomal component of the somites. Taken together, these studies have demonstrated that the SM22α promoter is an excellent model system with which to define the transcriptional regulatory programs that control SMC- and somite-specific gene expression. As such, elucidation of the *cis*-acting elements and *trans*-acting factors that control SM22α gene expression in arterial SMCs should facilitate future studies examining the molecular mechanisms that underlie SMC diversity and those that modulate the SMC phenotype.

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